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March 26, 1993

Judith A. Foster
Applied Biological Sciences Branch,
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APR 2 1993
PROCUREMENT

"Purification of Cellulases of Microbispora", (Subcontract No. XD-2-11201 under Prime Contract No. DE-AC02-83CH10093

Dear Ms. Foster,

Attached is the Final Contract Report and general associated documents.

This summary covers all details of the larst months' activities. Large scale production (50 liter) and purification of the cellulase is still under current development.

Sincerely,

Douglas E. Eveleigh
Professor of Microbiology

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March 26, 1993

Dr. Michael Himmel,
Applied Biological Sciences Branch,
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1617, Cole Blvd.,
Golden, CO 80401-3393 Fax No. 303-278-3692

"Purification of Cellulases of Microbispora", (Subcontract No. XD-2-11201 under Prime Contract No. DE-AC02-83CH10093

Dear Mike,

Attached is the Final Contract Report and general associated documents. Two purification approaches to obtaining cellobiohydrolase have been performed. Large scale production (50 liter) and purification of the several cellulase components is under current development.

The summary achievements of this contract include:

1. Continued maintenance of vigor of cultures of *Microbispora* through culture on oatmeal/yeast extract agar.

2. Fortuitous selection of a variant culture of *M. bispora* that secretes beta-glucosidase into the culture broth, in contrast to the original isolate in which this enzyme was strongly cell/membrane bound.

3. Hagerdahl/Pye medium with Solka floc as the carbon source, was shown to be the best of several media for optimal cellulase production.

4. A three step classical protocol developed for purification of CBH II.

5. Methodology and constructs made for developing an affinity purification scheme based on the use of a "nickel-ligand" column to give selective binding of heterologous proteins containing a

peptide sequence of histidine peptides, has been accomplished. However, the affinity purification trials with endoglucanase MbcelA, have not proved effective. It was useful that the enzyme was selectively transported to the periplasm, thus giving a considerable amount of purification.

I thank Steve for pointing out the problems with the proposed sequence of the cellobiohydrolase gene. Our latest trials with the CBH II gene in *E. coli* are positive. We have sent Steve Ap4 and the 8Kb fragments/ constructs that we had at hand.

Copies of the report are being to Judith Foster.

Sincerely,

Douglas E. Eveleigh
Professor of Microbiology

c.c. Judith Foster, Subcontract Administrator, NREL
David Rumbo, Rutgers Research & Sponsored Programs
Arleen Nebel, Rutgers Accounting Monitor

"Purification of Cellulases of Microbispora", (Subcontract No. XD-2-11201 under Prime Contract No. DE-AC02-83CH10093

ANNUAL TECHNICAL - MARCH 1993

To:

Dr. Michael Himmel,
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From:

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OUTLINE

ABSTRACT: A thermophilic actinomycete *Microbispora bispora* was selected for cellulase production as it gave good yields of a stable cellulase and its ß-glucosidase was resistant to end-product inhibition. The cellulase is comprised of endoglucanases, cellobiohydrolases and cellobiases that act synergistically. These components have been characterized to differing degrees. This report details the further characterization of this cellulase complex, with focus on gaining overproduction and purification of cellobiohydrolase II.

INTRODUCTION:

We have studied the cellulase of the thermophilic, actinomycete *Microbispora bispora*. This bacterium from New Jersey was selected as giving the greatest yields of cellulase from a range of isolates recovered from worldwide soils/composts/hot spots. It is representative of such Actinomycetes. By classical biochemical techniques the cellulase was shown to be comprised of thermostable, synergistically acting components: 4 endoglucanases, 2 exoglucanases (cellobiohydrolase) and ß-glucosidase. From these introductory studies, interest in the **M. bispora** cellulase system was shown to be three fold:

1. It is a good thermally stable robust and effective cellulase.

2. Cellobiohydrolases are key components of cellulases, yet bacterial cellobiohydrolases are not widely described. Interest in *M. bispora* cellobiohydrolases is therefore high. It is of interest to determine if the **M. bispora** cellobiohydrolases act in a signinficant manner and interact synergistically with other cellulase components from other bacterial species.

3. The beta-glucosidases show resistance to end-product inhibition and thus could be of interest in the production from cellulose of high concentration glucose syrups.

Cloning has permitted resolution of an endo-glucanase, a cellobiohydrolase and two ß-glucosidases as soluble components in *E. coli*. The genes for one endo-glucanase, and two beta-glucosidases have been sequenced, their enzymes characterized. One of these latter two enzymes one is clearly a cellobiase (Wright et al., 1992). The other was shown to be a glucan-glucohydrolase that acted preferentially towards oligodextrins rather than to cellobiose (A. Goyal, 1993). The role of this enzyme in cellulolyses either in combination with the other cellulase components or even acting above written the cell is still to be determined.

As bacterial cellobiohydrolases are relatively rare, this study evolved to focus on the cellobiohydrolase, especially preparation of purified enzyme.

Cellobiohydrolase II

Two cellobiohydrolases (I & II) were first purified from culture broth (Yablonsky et al., 1989). Subsequently the cellobiohydrolase gene CBHII was characterized (Hu et al., 1992). However CBH yields from the *E. coli* were extremely low. Indeed the original selection of this clone being based on the use of a sensitive monoclonal antibody assay. Greater expression (x20) was subsequently gained by subcloning into a *Streptomyces lividans* (Hu et al., 1993). Further characterization of the CBHII enzyme is presented.

A *M. bispora* genomic library was made via cloning in *E. coli* and a clone containing cellobiohydrolase II was selected using a specific monoclonal antibody, and the gene was cloned into *S. lividans*. The cloned CBH II hydrolysed cello-oligosaccharides to yield cellobiose as the major product (analysis by HPLC).

The cloned enzymes from *E. coli* and *S. lividans* have the same pH and temperature optima as that of the native form. A slight decrease in thermostability of the enzyme produced in *E. coli* compared to the native enzyme was suggested to be the result of glycosylation of the native form and non-glycosylation of the cloned enzyme. *M. bispora* CBH II cloned in *S. lividans* was partially purified and then characterized. The K_m and V_{max} towards pNPC were 1.18 mM and 1.32 µmole/min/mg respectively. Its pI was 3.95, the pH optimum 6.5, and the temperature optimum is 60°C. The greatest thermal stability was between pH 5.0 to 8.0. The cloned CBH II hydrolysed cello-oligosaccharides to yield cellobiose as the major product (analysis via HPLC).

Overproduction of Cellobiohydrolase:

Production of Cloned enzyme: A principle objective of the NREL Contract is to produce samples of pure enzymes. As noted above cellobiohydrolase was selected as the component of *M. bispora* cellulase of primary interest. The first decision was should the native or cloned enzyme be purified? As cloning in itself yields a cellulase in the absence of other ("contaminating") cellulase components, production of the enzyme was approached using an *E. coli* clone containing the CBH gene. Enzyme yields were low. Subsequently the gene was expressed from an actinomycete/*E. coli* shuttle vector in a Streptomycete host. This gave a 20 fold increase in yield, and the cellobiohydrolase was secreted (Hu et al., 1993). However, inspite of various constructs being made, overall yields were never very high. Alternate molecular biological approaches to gain pure CBHII were considered, one being to develop a specific affinity purification scheme based on the use of a "nickel-ligand" column to yield selective binding of heterologous proteins containing a peptide sequence of six histidine residues.

A trial system was run with constructs of MbcelA endo-glucanase, as this enzyme is readily monitored. Considerable time was spent on the methodology and making constructs for developing an affinity purification scheme based on the use of a "nickle-ligand column to give selective binding of heterologous proteins containing a peptide sequence of histidine peptides. The cloned endoglucanase MbcelA, was used and though affinity pruification was not achieved, rapid induction and selective location in the periplasm did occur. This aspect for development of affinity purification is detailed in appendix II.

At this stage it was considered that most practical course to gain purified CBH II was to produce the enzymes from the native organism and purify of the enzyme (from the culture broth) through classical approaches - see Appendix II.

Thermophilic bacteria can be difficult to culture and maintain. It is well known that many thermophilic actinomycetes have "just died out" with continued laboratory culture - quote by Mary Lechevalier (an expert on thermophilic actinomycetes). We have maintained our stock cultures in liquid nitrogen, and in a deep freeze (-80°C). However, periodically we note that cultures maintained on cellulose-salts medium become weak. Indeed, early in this year's project the bacterium could not be grown on agar medium, and was maintained by weekly culture in liquid medium. We assume that through this protocol, a variant was fortuitously selected that secretes some beta-glucosidase into the culture broth, in contrast to the original isolate in which this enzyme was strongly cell/membrane bound. It was of interest to maintain this strain, and a variety of solid culture media were tested for supporting the growth of **M. bispora**. Of several solid media evaluated, growth was only gained on oatmeal/yeast extract agar. This medium has greatly simplified maintenance of *Microbispora*, and we consider this an extremely useful discovery.

LARGE SCALE CULTURE FOR CELLOBIOHYDROLASE PRODUCTION:

Prior to to large scale culture, we evaluated a range of media in order to gain optimal cellulase production. Hagerdahl-Pye salts medium was originally used for cellulase production with Avicel as the carbon/cellulase inducing source. This gave good though not outstandingly high enzyme yields. Comparison was made with the Stutzenberger medium and with various cellulose substrates. Highest yields were gained with Hagerdahl-Pye medium with Solka floc as the carbon source.

Large scale (50 l) fermentation has been accomplished and from the purification trials, a practical purification protocol has been developed (Appendix I).

Appendix I. Purification of Cellobiohydrolase from Microbispora bispora.

(Yong Mei Wu [NREL support] & Dinesh Yernool)

The original purification protocols established by T. Bartley (Ph. D. thesis) were followed. The culture was grown on Solka Floc in stages for a final 50l batch. The culture broth was concentrated via a hollow fiber filter and then clarified by centrifugation prior to purification (see Table 1).

The initial ion exchange step (Fig. 1) yielded two cellobiohydrolases, four endoglucanases and two beta-glucosidases. Note also an endoglucanase and cellobiohydrolase in the wash through zone.

Two major differences to the Bartley results were noted.

1. The cellobiohydrolases now eluted with the endoglucanases: compare Fig. 1 (present data) with Fig 1a (prior data).

2. Small amounts of cell free beta-glucosidases were found: in our prior studies the beta-glucosidases had been tightly membrane bound and were never found in the broth supernatant. Prior efforts to solubilize the beta-glucosidase components from the walls/membrane were negative. Initial purification of the beta-glucosidases was set up as a side project: Dinesh Yernool.

Further purification of the cellobiohydrolase using Peak II (5 fractions from a 20 peak fraction) employed a P100 gel filtration column (Fig. 2). Some resolution of the cellobiohydrolase was obtained from the contaminating endoglucanase.

Further resolution of the CBH using a Mono-Q column with analysis via electrophoretic analysis showed that the CBH peak showed one major and one minor band (silver staining). The cellobiohydrolase was active (use of MUC in the gel) following IEF electrophoresis, and moved as a single band in the gel. Even so, two endoglucanases were present, one of which was nearly separated from the cellobiohydrolase (Fig. 3).

The yield of the CBH "II" was 70 micrograms from 1/5th of the concentrated and lyophilized culture broth. Greater amounts can be obtained as:

a. only 1/5th of the starting material was used (i.e. potentially 350 μg).
b. an optimal zone (1/4) from the peak (Fig. 1) was used for the second fractionation step (350 x 4 + 1,400 μg).

(in the third step, the full peak [8ml] was used to load the Mono-Q column).

The 50 L fermentation has been run twice. The first run was unsuccessful and the second run (on which these results are based) yielded 0.0156U CBHase/ml. A further 50L fermentation is underway and purification will follow the above protocol (outline appoarach see Table 1).

Beta-glucosidases:

As noted above (Fig. 1), two beta-glucosidases were found present in the culture broth. This was in contrast to the cell-bound status in the earlier fermentations. The subsequent initial purification of these native beta-glucosidases showed them not to correlate (electrophoretic mobility) with the cloned enzymes (Bgl A and Bgl B) (Wright et al., 1992; Anil Goyal Ph. D. thesis 1993). From Wright and Goyals' studies we know that *M. bispora* has a beta-glucosidase (Bgl B; Wright et al., 1992) and a glucose exo-splitting glucanase (Bgl A; Goyal, 1993). Following the appearance of the beta-glucosidases in the culture broth, we will have to decide if there are further beta-glucosidase genes. The electrophoretic differences are a broad guide, but we will also have to determine the degree of glycosylation, etc.

The release of the beta-glucosidases is an interesting finding for the **M. bispora** cellulase system.

Table 1. Purification of Cellobiohydrolase from *M. bispora*

	Units[1]	Protein[2]	Units/mg	Purification	Yield (%)
Ultrafiltration sample	109	440	0.248	1	100
FFQ - Wash	19.4	53	0.366	1.48	50.1
- Peak I	8.2	5.6	1.46	5.89	
- Peak II	27.0	11.36	2.38	9.6	
Peak II 43-47#	11.8	3	3.93	15.9	10.8
Ultrafiltration[3]	6.57	2.15	3.05	12.3	6.0
P-100[4]	5.84	1.33	4.4	17.7	5.35
Mono Q	3.30	0.541	6.1	24.6	3.03

Notes:

1. Assay Conditions: Appropriately diluted enzyme incubated with pnpc (1mm) in pc buffer (ph 6.5) at 60°C for 15 min.
2. Bradford Microassay, using BSA as standard.
3. Amicon ultrafiltration unit, membrane YM10 (10,000 cut off).
4. Biogel P-100, Mono Q anion exchange chromatographies for Peak II fraction 43-47 only.

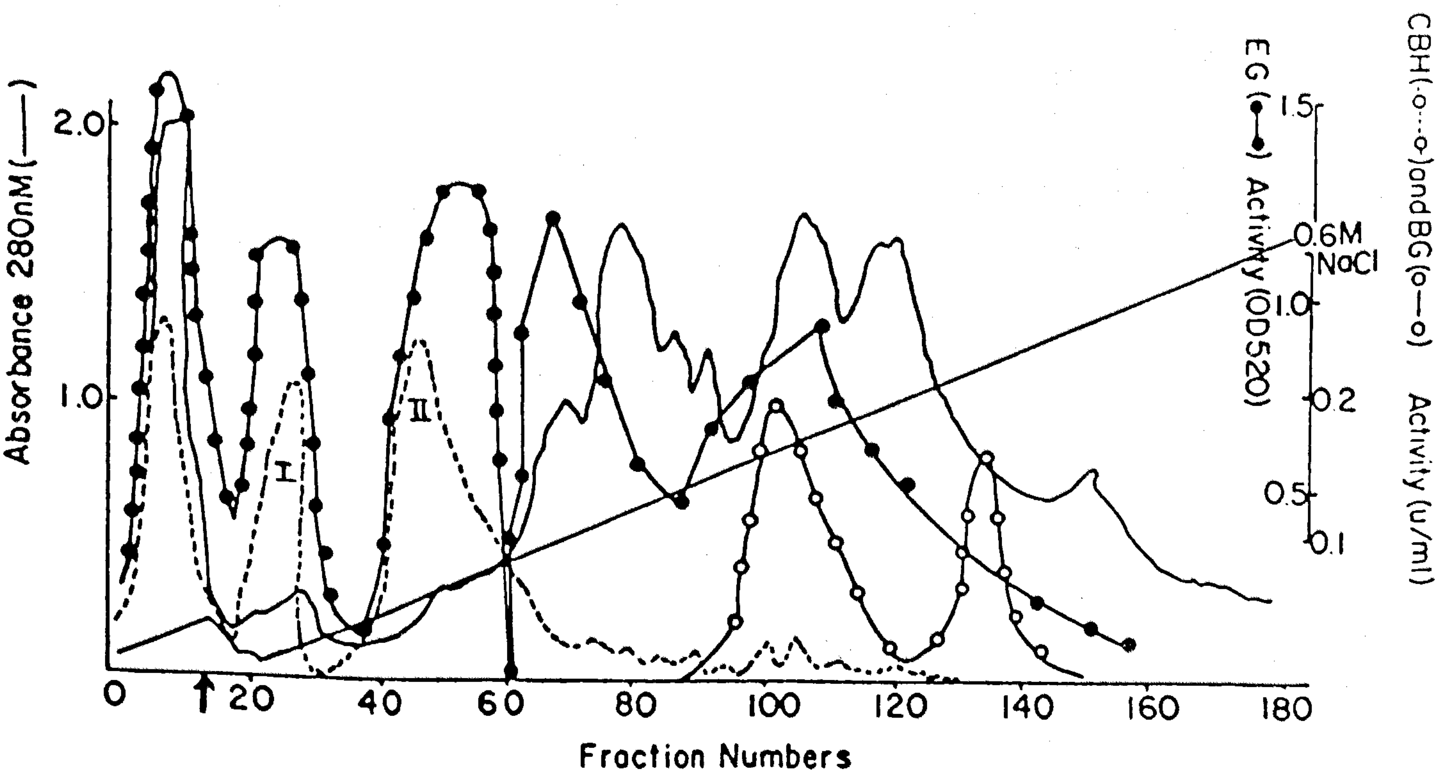


Fig. 1. Fractionation of M. bispora Cellulase Components - Ion Exchange (Fast Flow Q Resolution)

Fermenter broth was lyophilized and desalted eUltrafiltration - YM10 membrane) and a sample loaded onto a Fast-flow Q column (60 ml 0.01M Tris-HCl, pH 7.5). Elution was with a linear NaCl (0.6M) gradient. CBH and ß-glucosidase activity were assayed by using pNPC (1mM) and pNPG (1mM) as substrates respectively (60°C, 15 min). Endoglucanase activity was assayed with CMC (0.75%); 7L Hercules, Inc., Wilmington, DE) at 60°C, 30 min. and monitored using Somogyl and Nelson reducing sugar assay.

Cellobiohydrolase - - - - -

ß-Glucosidase —o——o—

Endoglucanase —●——●—

OD280 ————

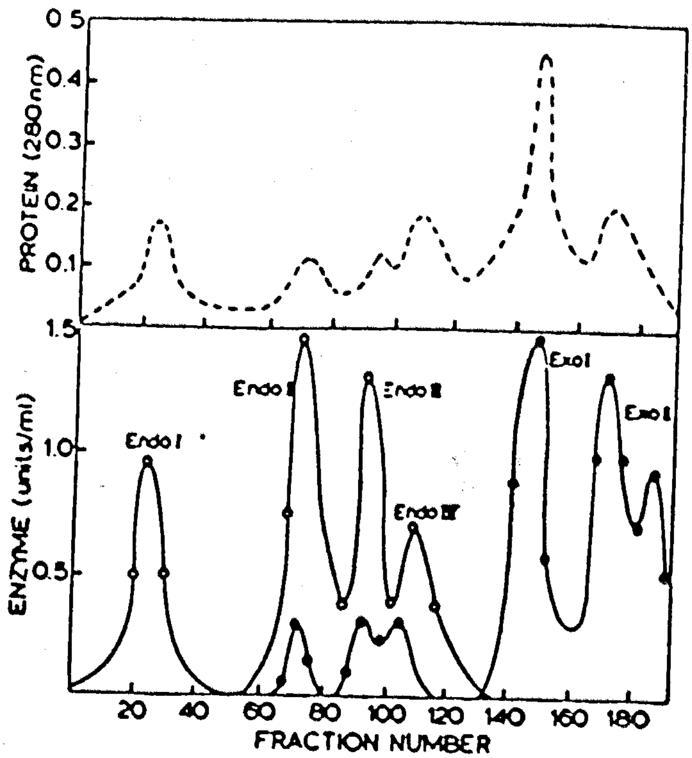


Fig. 1a. Resolution of M. bispora endo- and exoglucanase via ion-exchange chromatography (DEAE-Sepharose CL-4B).

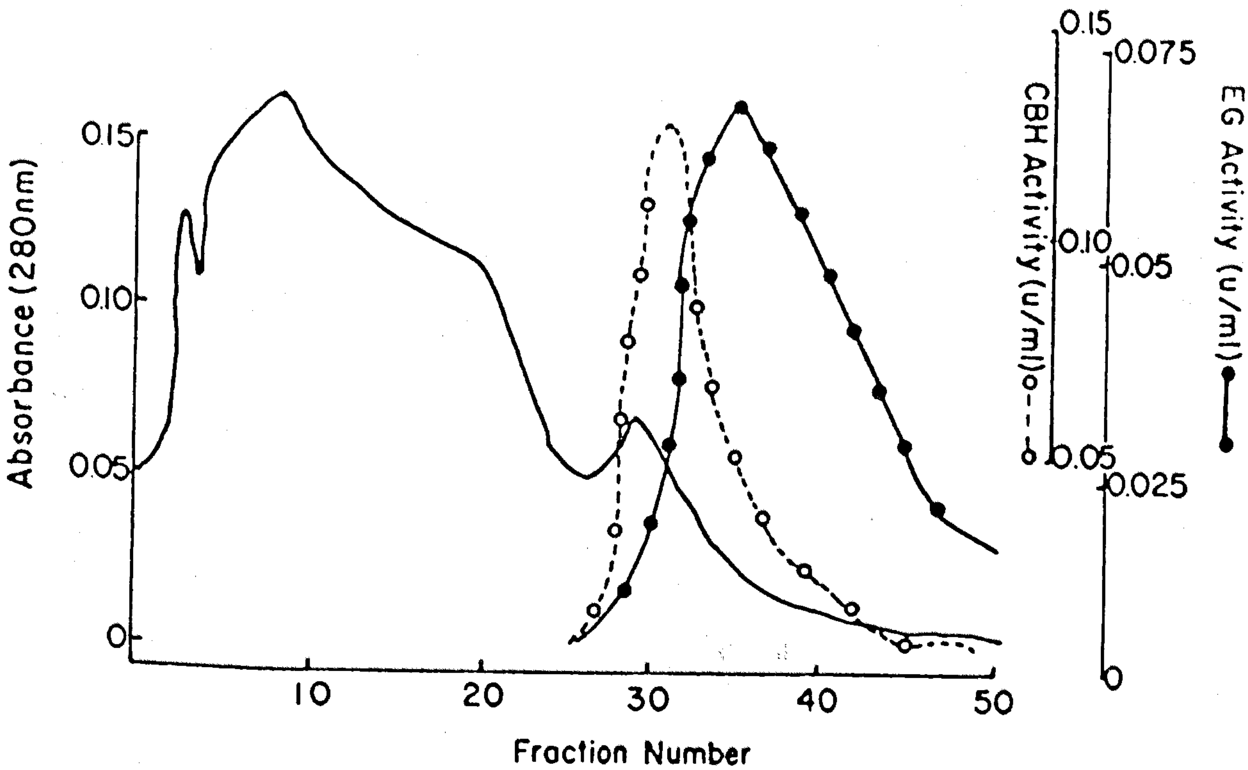


Fig. 2. Fractionation of Cellulase Components by Gel Filtration Chromatography (P-100 polyacrylamide).

Samples (1ml) were loaded onto a P-100 column (80 ml) and protein eluted with Tris-HCl (0.01M, pH 7.5). Fractions collected = 12 ml/hr. For details of the cellobio-hydrolase and endoglucanase assays see text and Fig. 1.

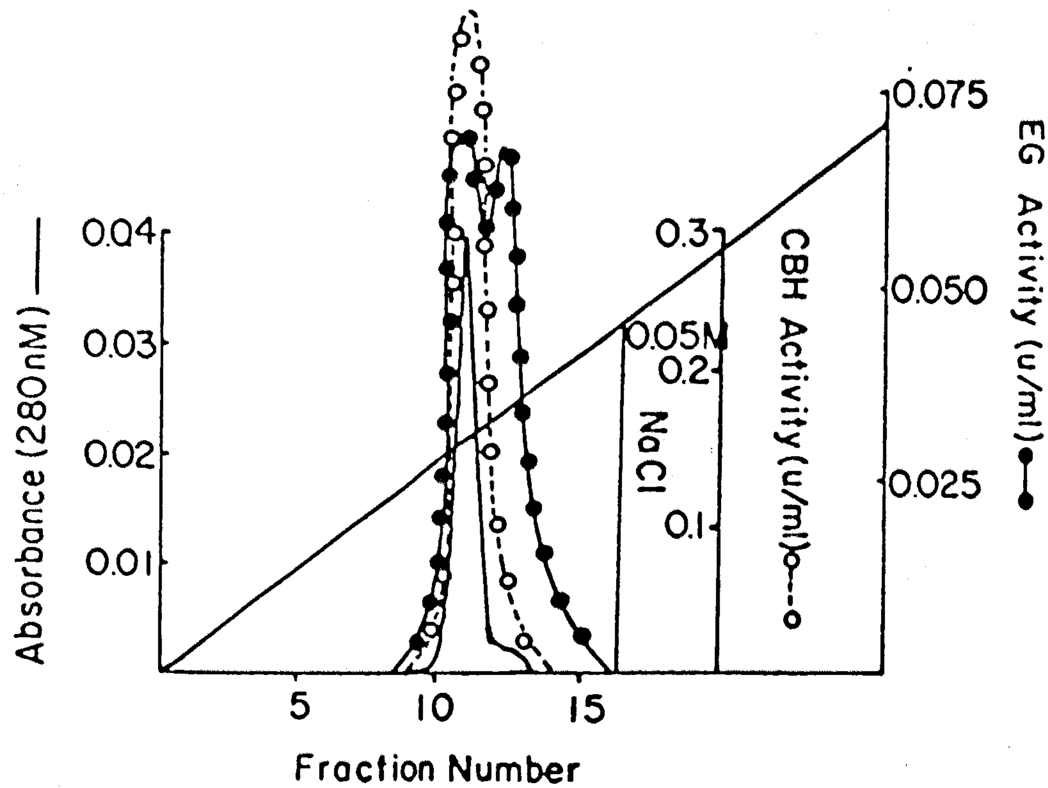
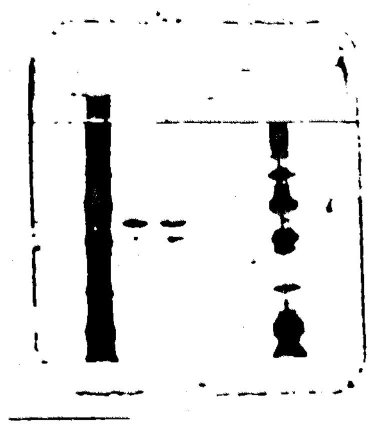


Fig. 3. Fractionation of Cellobiohydrolase and Endoglucanase by Ion-Exchange chromatography (Mono Q column).

Samples were loaded onto a Mono Q column (0.01M Tris-HCl, pH7.5). Protein was eluted with a linear NaCl (0.1M) gradient. For details of the CBH and EG assay details see text and Fig. 1.



1 2 3 4

Fig. 4. Phast gel SDS electrophoresis

Lanes:

1 & 4. Molecular weight markers

2. CBHase from a Mono Q column (Fig.3)

3. CBHase from P100 column (Fig 2)

DISCUSSION:

The general goals of characterization of the *M. bispora* cellulase have been achieved: there are four endoglucanases, two cellobiohydrolases and two ß-glucosidases. The genes of CBHII, endoglucanase A and the two ß-glucosidases (one a novel exo-glucosidase) have been sequenced, and characterized. Their position in the "classical" cellulase family tree has been established.

The original finding of a true CBH is noteworthy, as though CBH's occur widely in fungi and a few bacteria, a CBH has yet to be described in other actinomycetes. CBH is critical in the synergistic action of fungal cellulases, and could have similar importance in actinomycetes and also other bacteria. Further characterization of the *M. bispora* CBH and evaluation of its synergistic potential should prove fruitful.

The overall cloning approach to study the *M. bispora* cellulases has been particularly productive. For example, for the ß-glucosidases the understanding has gone from a membrane entrenched form in the native organism, to now showing that there are two distinct genes that code for unique enzymes. These latter two forms are soluble, and thus cloning has considerably facilitated their subsequent purification and characterization. The availability of clones of the three cellulase types, cellobiohydrolase, endo-glucanase and beta-glucosidase permits more indepth studies, such as deciphering the basis of the synergism that occurs between the components (now available as pure genetic entities), and analysis of the mechanism of end product inhibition of the ß-glucosidase.

The summary achievements of this contract include:

1. Continued maintenance of vigor of cultures of *Microbispora* through culture on oatmeal/yeast extract agar.

2. Fortuitous selection of a variant culture that secretes beta-glucosidase into the culture broth, in contrast to the original isolate in which this enzyme was strongly cell/membrane bound.

3. Hagerdahl/Pye medium with Solka floc as the carbon source, was shown to be the best of several media for optimal cellulase production.

4. Three step classical protocol developed for purification of CBH II.

5. Methodology and constructs made for developing an affinity purification scheme based on the use of a "nickle-ligand column to give selective binding of heterologous proteins containing a peptide sequence of histidine peptides.

Production of "semi-purified CBH" in a semi-purified state by its being located in the periplasm.

Publications via NREL and DOE Support:

Support has been in part for Y. Mei Wu, A. Goyal and P. Hu.

Goyal, A.K., R.M. Wright, P. Hu and D.E. Eveleigh 1992. Cellulase producing organisms: Developments through Recombinant DNA Technology. pp. 761-769, Proc. Ann. Auto. Technol. Devel. Mtg., publ. Soc. Auto. Engineers, Warrendale, PA.

Hu, P., T. Chase, Jr. and D. E. Eveleigh. 1992. Cloning of a cellobiohydrolase gene in *Streptomyces lividans*. Appl. Microbiol. Biotechnol. In press.

Hu, P., S. K. Kahrs, T. Chase, Jr. and D. E. Eveleigh. 1992. Cloning of a *Microbispora bispora* cellobiohydrolase gene in *Escherichia coli*. J.Indust. Microbiol. 10:103-110.

Wright, R.M., M.D. Yablonsky, Z. Shalita, A.K. Goyal and D.E. Eveleigh. 1992. Cloning, characterization and nucleotide sequence of *Microbispora bispora* Bgl B: a thermostable beta-glucosidase expressed in *Escherichia coli*. Appl. Environ. Microbiol. 58:3455-3465.

Presentations 1992:

Goyal, A.K., P. Hu, R.M. Wright, K.O. Elliston and D.E. Eveleigh. 1991. The cellobiohydrolases and beta-glucosidases of *Microbispora bispora*. Amer. Soc. Microbiol., Conf. Biotechnol. New York, NY.

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Wright, R.M., P. Hu, J. Bok, A.K. Goyal and D.E. Eveleigh. 1991. Thermophilic cellulases: *Microbispora bispora* and *Thermotoga maritima*. Gordon Conf. Appl. Environ. Microbiol., New London, NH.